APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1992, p. 353–358
0099-2240/92/010353-06$02.00/0

Vol. 58, No. 1

Isolation of 2,4-Diacetylphloroglucinol from a Fluorescent Pseudomonad and Investigation of Physiological Parameters Influencing Its Production

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Received 8 May 1991/Accepted 15 October 1991

***Pseudomonas* sp. strain F113 was isolated from the rhizosphere of sugar beets and shown to inhibit a range of plant pathogenic fungi by producing an antibioticlike compound. An antibiotic-negative mutant strain, F113G22, was generated by transposon mutagenesis. This mutant has lost the ability to inhibit both bacterial and fungal microorganisms on high-iron medium. The antibioticlike compound was subsequently identified as 2,4-diacetylphloroglucinol (DAPG), and a high-pressure liquid chromatographic assay was developed for to detect it quantitatively in growth culture media and soil. The growth temperature had a direct bearing on DAPG production by strain F113, with maximum production at 12°C. The iron concentration, pH, and oxygen had no influence on DAPG production by strain F113 under the assay conditions used. However, a low ratio of culture volume to surface area available to the microbe in the growth container was critical for optimum DAPG production. Different types of carbon sources influenced DAPG production by strain F113 to various degrees. For example, sucrose, fructose, and mannitol promoted high yields of DAPG by strain F113, whereas glucose and sorbose resulted in very poor DAPG production.**

Certain fluorescent pseudomonads from soil have been shown to promote plant growth by inhibiting bacteria and fungi that are deleterious to plants (14, 18, 27, 28, 32). The production of antibiotic substances by some strains has been recognized as a major factor in the suppression of many root pathogens. To date a number of these disease-suppressive antibiotic compounds have been characterized chemically and include N-containing heterocycles such as phenazines (4, 8, 31), pyrrol-type antibiotics (11, 12, 15), pyo-compounds (13), and indole derivatives (36). A small number of antibioticlike compounds that do not contain nitrogen have also been isolated from fluorescent pseudomonads (16); among these compounds is 2,4-diacetylphloroglucinol (DAPG), which has been used in the control of plant root diseases (10, 18). However, the non-N-containing compounds are a minor class, and work in this area represents only a small proportion of the total work on existing pseudomonad antibiotics.

To date these disease-suppressive compounds have been detected primarily by in vivo bioassays with indicator organisms (6). However, a high-performance liquid chromatography (HPLC)-based method could detect an antibiotic both qualitatively and quantitatively in situ. Such a method was recently employed by Thomashow et al. (31) for the detection of phenazine carboxylic acid in soil. This method can be very fast and can also determine whether other strains produce the same antibiotic. Another major advantage is that accurate quantitative measurements of a specific antibiotic can be obtained.

The production of antibiotic compounds by fluorescent *Pseudomonas* spp. is influenced by different chemical conditions. Douglas and Gutterson (6) showed that glucose stimulated the production of some antibiotics by a strain of *Pseudomonas fluorescens*, whereas the production of other antibiotics was inhibited by glucose. The production of other pseudomonad metabolites is also influenced by growth conditions. For example, the production of siderophores (iron-binding ligands) is regulated by iron (21) and also by temperature (7).

In this study, we report on the isolation of a fluorescent *Pseudomonas* strain that can inhibit a number of plant root pathogens by producing DAPG. DAPG is a non-nitrogen-containing compound and is one of just three phloroglucinols that have been isolated from fluorescent pseudomonads. We describe the isolation of this compound and also the development of an HPLC assay for its quantitative detection in both culture media and soil. We used this assay to test the effect of different environmental conditions on the production of DAPG by this strain.

MATERIALS AND METHODS

Organisms and culture conditions. *Pseudomonas* sp. strain F113 was isolated from the root hairs of a sugar beet plant. This strain is a fluorescent, gram-negative, nonfastidious motile rod that is oxidase and catalase positive, indicating a member of the *Pseudomonas* species as outlined in *Bergey's Manual of Determinative Bacteriology* (2). It was cultured on minimal sucrose asparagine (SA) medium (26) at 28°C. On this medium it produced a fluorescent yellow-green siderophore after 1 day and a brown pigment after 4 days. Strain F113G22 is an antibiotic-negative mutant of strain F113, and its construction is described below. F113G22 was maintained on SA medium containing kanamycin (10 μg/ml). The plant pathogenic fungi *Pythium ultimum*, *Phoma beta*, *Rhizopus stolonifer*, and *Fusarium oxysporum* were obtained from the Commonwealth Mycological Institute, Surrey, United Kingdom, and maintained on potato dextrose agar (PDA). *Escherichia coli* SM10, which has the chromo-

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somally inserted RP4-2-7C::Mu (29), was cultured on Luria-Bertani (LB) medium (19) at 37°C.

Transposon mutagenesis. *Pseudomonas* sp. strain F113 was mutated with Tn*5-lac* by using the delivery plasmid pSUP101::Tn*5-lac* (20). *E. coli* SM10 was the donor strain, and mutagenesis was carried out as described previously (21).

Strain fingerprinting. To confirm strain identity, the membrane proteins of mutant strains grown under low-iron (no exogenous iron added) and high-iron (50 μM $FeCl_3$) conditions were isolated and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (21). The protein profile was compared with those of the parent and control strains.

Isolation of crude antibiotic compound. The bacterial strain F113 was inoculated on SA semisolid agar (0.35%) plates and incubated at 28°C for 8 days. The total contents of 12 plates were placed in a 1-liter Duran flask (Schott:Mainz, Germany), to which was added 250 ml of 80% aqueous acetone. The flasks were shaken on a gyratory shaker (model G10; New Brunswick Scientific Co., Inc., Edison, N.J.) at 200 rpm for 24 h at room temperature, and the contents were then centrifuged at 15,000 rpm on a Beckman JA-10 rotor for 20 min at 10°C. The supernatant was decanted and condensed by removal of the acetone in vacuo at 45°C. After the aqueous concentrate was filtered under vacuum through a filter (pore size, 0.45 μm), 20-ml portions were extracted twice with 2.5 volumes of diethyl ether. The ether extracts were taken to dryness in vacuo, and the residue was extracted with 30 ml of acetone, to yield a white cloudy suspension. Methanol was added dropwise until a white solid precipitated. The liquid was filtered through a 0.45-μm-pore-size filter and condensed in vacuo to approximately 2 ml.

Purification. Two milliliters of the crude isolate was streaked on thin-layer plates coated with silica gel G.F. (Sigma Chemicals Ltd.) and developed in dichloromethane-hexane-methanol (50:40:10, vol/vol). The plates were observed under long- and shortwave UV light for fluorescing and absorbing bands. All bands and blank areas were removed separately from the plates and eluted with acetone (50 ml). After the detection of active bands by using the fungal inhibition assay, the acetone eluate was taken to dryness in vacuo and taken up in hot hexane (10 ml), and the antibiotic was precipitated from it by slow cooling.

Fungal inhibition assay. Aliquots (0.1 ml) of the acetone eluates were placed directly onto SA agar plates and placed under a laminar-air flow hood until the acetone had evaporated. PDA plugs of *P. ultimum* were placed ~3 mm from the edge of the residue, and the plates were incubated at 28°C for 2 days. Inhibition was determined by prevention of growth in the direction of the residue. For fungal inhibition assays with *Pseudomonas* strains, the test strain was streaked 2 cm around a PDA plug of the fungus that was placed in the center of the plate. Inhibition of *E. coli* was carried out by overlaying *Pseudomonas* colonies on an LB agar plate with a freshly grown *E. coli* culture in semisolid LB agar medium. The plates were incubated at 28°C for 48 h before they were scored.

HPLC apparatus. The HPLC system consisted of a Waters pump (model 510), a rheodyne injection valve with a 20-μl loop, and an analytical column (150 mm by 4.5 mm [inner diameter] by 6 mm [outer diameter]) packed with octadecylsilica (Hypersil; particle size, 5 μm). The spectrophotometric detector (model 440) was from Waters Associates and was linked to a Phillips 8251 strip chart recorder, which plotted the detector outputs. The reagents for mobile-phase preparation were of HPLC grade, and all mobile phases used were filtered and degassed on a Millipore HPLC filtration system with 0.45-μm-pore-size membrane filters. All samples were run at a flow rate of 1.0 ml/min and detected at a wavelength of 254 nm.

Sample pretreatment method for HPLC assay. Because of the inherently complex nature of the sample, which included medium constituents and a range of secondary metabolites, a sample pretreatment method with solid-phase extraction was developed. Advantage was taken of the retention behavior of the antibiotic on octadecylsilica. The antibiotic was retained on a Sep-Pak C_{18} cartridge when applied in water as the solvent. The cartridge was conditioned by flushing with 10 ml of methanol and then 10 ml of H_2O, and this procedure was repeated three times. To obtain a supernatant from solid agar plates, the agar was first pulverized and then centrifuged at 10,000 rpm for 15 min, and the supernatant was decanted. Then 5 ml of filter-sterilized culture supernatant was injected onto the cartridge, which was washed with 30 ml of H_2O and then with 20 ml of methanol to elute the antibiotic. The methanol eluate was taken to dryness in vacuo, the residual material was dissolved in 5 ml of the mobile phase, and 20-μl aliquots were injected into the HPLC system.

To evaluate the effect of soil on the detection of DAPG via the HPLC assay, purified DAPG (in ether) was mixed into the soil at concentrations of 100, 50, and 25 μg/g of soil. The soil used (pH 6.9) was taken from the upper 5 cm of the soil profile, sieved through a 0.5-cm-mesh screen, and air dried before use. The ether was removed in vacuo at 35°C, giving a uniform dispersion of DAPG throughout the soil. To measure the amount of recoverable DAPG, the soil was washed repeatedly in diethyl ether. The washings were pooled and dried under vacuum at 30°C, and the residue was reconstituted in the mobile phase and injected into the HPLC system.

Effect of different growth conditions on DAPG production. The amount of surface contact in the growth container was increased by adding different amounts of granite chips 1 to 2 mm in diameter. These were prewashed twice in alcohol and water and autoclaved before they were added to each of 15 50-ml Erlenmeyer flasks containing 15 ml of SA broth. The effect of agar concentration on DAPG was studied by using SA incorporating different agar concentrations. The agar preparations were sterilized by autoclaving, and 20-ml volumes were poured into sterile petri dishes. In all cases, the inoculum used was 4% of an overnight culture that was spread plated for solid media and incubated stationary at 28°C for 8 days. To test the effect of temperature on DAPG production, four 100-ml Erlenmeyer flasks each containing 5 ml of SA broth were inoculated with strain F113 and incubated with shaking at 28°C for 18 h. The flasks were removed and incubated stationary at the desired temperatures. To investigate the effect of oxygen on DAPG production, 71-ml serum bottles with 2 ml of SA broth were used. For growing cells, each bottle was inoculated with ~10^3 CFU/ml, whereas for stationary-phase cells an inoculum of ~10^9 CFU/ml was added. In each case, the gas phase in the assay flasks was composed of the desired percentage of oxygen and the remainder was made up with argon. To check the carbon source, pH, and iron concentration most suited to antibiotic production, 80 mM solutions of a range of carbon sources were prepared and buffered with inorganic salts K_2HPO_4, KH_2PO_4, and NH_4Cl (0.004, 0.0022, and 0.0049 M, respectively). $MgSO_4 \cdot 7H_2O$ (0.05%) was added

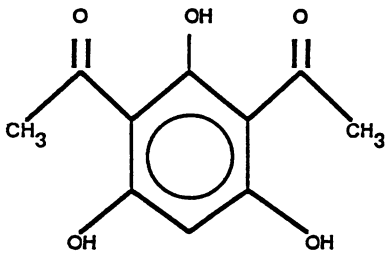

FIG. 1. Structure of DAPG.

after autoclaving. For pH and Fe analysis, sucrose was the carbon source of choice. The pH was adjusted with 2 N HCl and 2 N NaOH across the pH range 2 through 10. Iron-rich conditions were obtained by adding 10, 50, 100, and 200 μM $FeCl_3$, and iron-poor conditions were obtained by adding 0, 10, and 100 μM ethylenediamine-di(O-hydroxyphenyl)acetic acid. In all cases, approximately 10^9 CFU were added per ml of test solution. The cells were pregrown to the stationary phase in SA medium, washed three times in Ringer solution (Oxoid Chemicals), and incubated stationary at 12°C. All samples analyzed for antibiotic production were pretreated as outlined above for the solid-phase extraction and HPLC assay procedures, and the antibiotic production profiles were recorded after 4 and 8 days.

RESULTS

Characterization of *Pseudomonas* sp. strain F113. Strain F113 did not produce a fluorescent siderophore during growth on SA medium containing 50 μM $FeCl_3$. A brown pigment was produced after approximately 4 days of growth on both low-iron (no exogenous iron added) and high-iron (50 μM $FeCl_3$) media. The ability of the strain to inhibit the growth of both bacterial and fungal organisms was tested on this medium. The growth of *E. coli* and the plant pathogenic fungi *P. ultimum*, *P. beta*, *R. stolonifer*, and *F. oxysporum* was inhibited by strain F113.

Construction of antibiotic-negative mutant strain F113G22. Strain F113 was mutated with the transposable element Tn*5-lac*, and Km^r colonies were spot inoculated onto LB agar medium (50 colonies per plate) for inhibition assays with *E. coli* as the test strain. One colony was obtained (out of approximately 1,000 screened) that did not inhibit the test organism and was designated strain F113G22. Further characterization revealed that it had also lost the ability to inhibit fungi on high-iron (50 μM $FeCl_3$) medium. Under these conditions, wild-type strain F113 completely retarded growth of *P. ultimum*, whereas mutant strain F113G22 had no effect on the proliferation of the fungus, which completely covered the surface of the agar. It was also noted that this mutant did not produce the brown pigment. However, on low-iron medium the production of siderophore still enabled it to inhibit some test organisms, e.g., *P. ultimum*. It was confirmed that the mutant was derived from the wild-type strain by comparison of the membrane protein profiles from both strains. The membrane proteins that were separated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis were identical in both cases and differed from the membrane protein profiles of other selected pseudomonads (data not shown).

Antibiotic identification. The mass spectrum of the isolated compound was matched to that of ethanone 1,1-(2,4,6,trihydroxy-1,3-phenylene)bis, the trivial name of which is DAPG (Fig. 1). This, in conjunction with ^1H and ^{13}C nuclear magnetic resonance, infrared spectra, and melting point and elemental analyses (data not shown), confirmed the structure indicated by the mass spectrum data.

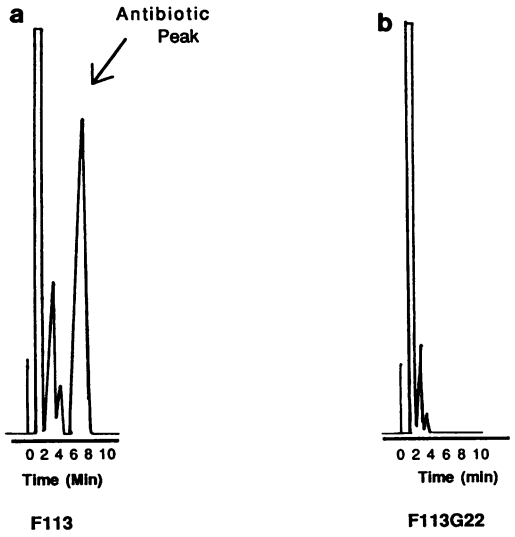

FIG. 2. HPLC chromatographic profiles of culture supernatants from strains F113 (a) and F113G22 (b), with no prior solid-phase extraction.

Confirmation that the antibiotic activity of strain F113 is due to DAPG. The extraction procedure outlined above was applied to the mutant strain F113G22. Crude extracts of F113G22 and F113 were subjected to thin-layer chromatography. The active band of strain F113 was not present in the mutant strain F113G22, and the acetone-methanol crude isolate of the latter strain showed no inhibition properties. This was further substantiated by recording the HPLC profiles of the initial centrifuged supernatants from strains F113 and F113G22 without prior solid-phase extraction. The DAPG peak of strain F113 was not present in the mutant strain F113G22 (Fig. 2).

Physiological parameters influencing production of DAPG by strain F113. The effect of a number of parameters on the

TABLE 1. Effect of oxygen on antibiotic production by strain F113[a]

[Oxygen] (%)	[DAPG] (μg/liter/10^5 CFU)	
	Growing cells[b]	Resting cells[c]
0	ND	0.03
2	3.61	3.84
3	3.90	4.30
4	4.02	3.84
5	4.13	4.69
6	3.46	5.32
8	3.95	4.36
10	4.21	4.10
15	3.72	4.23
20	4.02	4.16

[a] The values shown were recorded after 8 days. ND, not detected.
[b] Cells were grown under the stated oxygen levels with an initial inoculum of 10^3 CFU/ml.
[c] Resting cells (10^9 CFU/ml) pregrown under the atmospheric oxygen conditions used in each case.

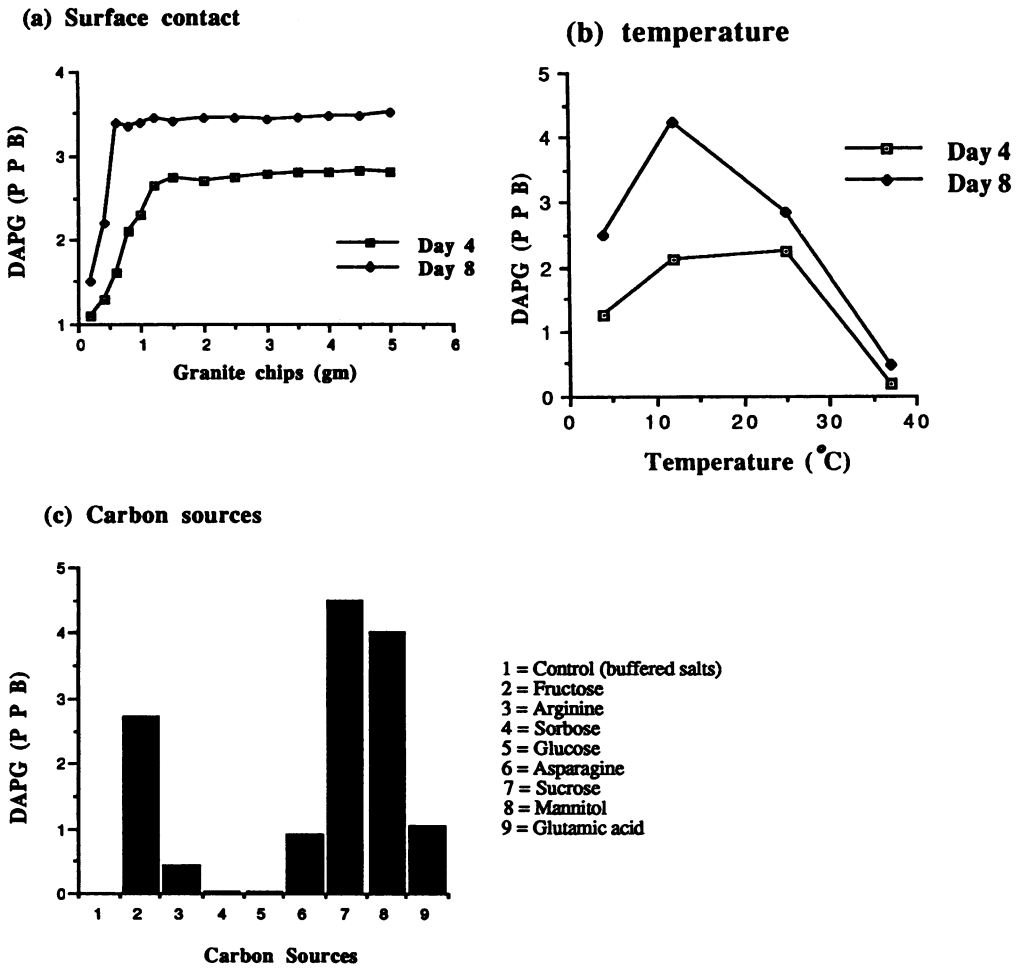

FIG. 3. Effect of surface contact (a), temperature (b), and carbon source (c) on DAPG production by strain F113. Measurements in panel c are after 8 days of incubation. All measurements are the means of three independent experiments. The DAPG concentration was standardized for 10^5 CFU.

production of DAPG by strain F113 was studied. Strain F113 grown in 1-liter Duran flasks containing large volumes of SA broth (>600 ml) produced negligible quantities of antibiotic. However, when strain F113 was grown in volumes of SA broth such that the liquid depth was less than 1 cm, the amount of antibiotic produced was greatly increased (>500%). Similar quantities of DAPG were also recorded from strain F113 grown on semisolid agar. Monitoring antibiotic production by strain F113 on both semisolid SA agar and in small volumes of SA broth showed that maximum antibiotic production occurred between 4 and 8 days, with negligible increases thereafter.

The above observation prompted us to investigate the role of oxygen availability on DAPG production. Lower percentages of oxygen supplied to the growing cells resulted in a significant decrease in the number of F113 cells present and thus in the amount of antibiotic detected. However, when DAPG concentration was correlated with the number of CFU in the cell suspension, no significant variation was observed with different oxygen levels supplied in the gas phase (Table 1). Similarly, when resting cell suspensions pregrown at normal aerobic conditions were exposed to various oxygen levels for 8 days, no apparent differences in DAPG production were observed (Table 1). However, when no oxygen was added to the gas phase, a significant reduction in DAPG production occurred.

It is also possible that the ratio of the culture volume to the total surface area in a growth container affects the production of DAPG by strain F113. This was investigated by adding increasing amounts of agar to a broth culture, thus increasing the viscosity, and monitoring the effect on antibiotic production. When the agar concentration was increased from 0 to 1.5%, the amount of antibiotic produced per 10^5 CFU increased from 0.46 to 5.21 ppb. However, further increases in agar concentration did not significantly affect DAPG concentration. In addition, increasing the available surface area by sequentially adding sterilized 1- to 2-mm granite chips to a broth culture resulted in a corresponding increase in DAPG production (Fig. 3a). This effect did not increase indefinitely, suggesting that there is a minimum amount of surface contact required for optimum DAPG production.

To test the effect of temperature on DAPG production by strain F113, pregrown cells were incubated at temperatures ranging from 4 to 37°C. Optimum DAPG production occurred at ~12°C (Fig. 3b). Negligible DAPG production occurred at 37°C, even though all cells remained viable.

Other parameters in soil that are known to affect microbial

metabolite production are pH and iron concentration. However, adding iron concentrations in the range 0 to 200 μM $FeCl_3$ did not effect DAPG production by pregrown cells of strain F113 under the assay conditions used. Similarly, when pregrown cells were resuspended in assay solutions ranging in pH from 2 to 10, there was no change in DAPG produced per viable CFU. It was noted that at both extremes of pH no viable cells remained after 4 days.

Different carbon sources influenced the production of DAPG by strain F113. When pregrown cells were incubated in the presence of sugars and amino acids, fructose, sucrose, and mannitol promoted high yields of DAPG, whereas no DAPG production was observed when cells were incubated in glucose and asparagine, even though all cells remained viable (Fig. 3c). This indicates that the type of carbon source greatly influences the production of DAPG by strain F113.

DISCUSSION

Pseudomonas sp. strain F113 inhibited both bacterial and fungal organisms during growth in low-iron and high-iron (50 μM $FeCl_3$) conditions, indicating that its antibiosis is not due solely to the production of a fluorescent siderophore. We obtained a Tn*5*-induced mutant (strain F113G22) that no longer inhibited *P. ultimum* or *E. coli* in high-iron conditions, and so we assumed that the parent strain produced only one antibiotic compound under these conditions. However, the possibility that other phloroglucinol derivatives or structurally related compounds are produced cannot be discounted. A number of phloroglucinol derivatives have been detected in a single fluorescent *Pseudomonas* strain (5) and also in *Aeromonas hydrophila* (30). This antibiotic compound was identified as DAPG. This non-nitrogen-containing compound has been isolated from fluorescent pseudomonads (5, 10, 23) and *A. hydrophila* (30), and a number of studies on its antibiotic properties have been published. Katar'yan and Torgashova (17) reported on some herbicidal effects of DAPG, and Reddi and Borovkov (24) studied its activity against a range of bacteria and fungi. Surprisingly these authors found that DAPG was very active against gram-positive bacteria, but that fungi and many gram-negative bacteria, including *E. coli*, were relatively insensitive. This contrasts somewhat with our results; we found DAPG to be very active against *E. coli* and a range of plant pathogenic fungi.

The production of DAPG by bacteria has been detected by using bioassays (25, 30). However, although these assays indicate the presence of an inhibitory substance, they do not specify the nature of the compound or the amount produced. To develop a rapid method for detection of DAPG production in vivo, a simple HPLC-based method was developed. This assay shows the presence of DAPG both qualitatively and quantitatively in culture supernatants. Phloroglucinols have previously been investigated chromatographically by using gas-liquid chromatography (22) and HPLC (34). However, in this study, we modified the HPLC procedure for the detection of a particular phloroglucinol (DAPG), incorporating a sample pretreatment step that eliminates non-C_{18}-retained material.

The amount of DAPG produced was affected by different growth conditions employed. However, other conditions examined, such as pH and iron concentration, had no effect on its production under the assay conditions used. The optimum temperature for DAPG production was ~12°C, indicating that soil temperature is conducive to maximum antibiotic production. This differs from the results from the strain studied by Bolton and Elliot (3), who found that toxin production from a soil pseudomonad was not strongly influenced by temperature. Strain F113 produced small quantities of DAPG unless the ratio of surface area to volume was increased. One of the experiments supporting this conclusion involved the inclusion of granite chips in the growth container. In this attempt to simulate natural soil conditions, one cannot eliminate the possibility that certain minerals leached from the chips influenced DAPG production. However, the experiment further suggests that strain F113 may be required to attach to a solid surface for optimum DAPG production. It is also possible that the bacteria need to form microcolonies to produce significant quantities of DAPG. However, additional work is required to evaluate this aspect. All physical parameters examined (temperature, oxygen, and surface contact) indicate that the physical parameters of soil should allow maximum production of DAPG by strain F113. The parameters conducive to antibiotic production in soil have been reviewed (9, 33, 35), and the lack of specific nutrients is recognized as a major factor in limiting antibiotic production. Soil treated with various nutrient sources, e.g., fragments of straw, support antibiotic production (37). However, microorganisms often display a preference for specific carbon sources for the production of particular secondary metabolites (1, 6). In this study, DAPG production by strain F113 was promoted by some sugars, whereas others were ineffective for this purpose. These results suggest that optimum DAPG production by strain F113 in soil should be influenced by the availability of sucrose, fructose, or mannitol.

In conclusion, antibiotic production by fluorescent *Pseudomonas* spp. is now recognized as an important feature in plant disease suppression by some strains (10, 31). However, due to the scarcity of nutrients in most soils, antibiotic production is generally restricted. In this study we have demonstrated the selective enhancement of an antibiotic from a fluorescent pseudomonad by certain carbon sources. These results strongly suggest that prior knowledge of plant exudate composition and the ability of different carbon sources to induce a particular antibiotic are essential in selecting suitable bacterium-plant combinations for biocontrol purposes.

ACKNOWLEDGMENTS

We thank John Meehan and Pat Higgins for their technical assistance, Peter Caplin, University College, Dublin, for running the mass spectrum, and Peter Stephens for his helpful advice.

This work was supported in part by grants from the Irish Science and Technology Agency (ST/Bio/335/88 and ST/008/89), and European Community [ECLAIR-AGRE-0019-C and BAP-0413-C-(EDB)].

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